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Investigating the Role of HOXC10 as a Mediator of Metastasis in Breast Cancer

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14. ABSTRACT The overall purpose of this project is to investigate the role of HOXC10 in breast cancer tumorigenicity and drug resistance. Since it was previously shown to be involved in proliferation and cell cycle, we investigated at the molecular level the role of HOXC10 and found that by affecting the RB/E2F1 pathway, it controls proliferation, G1/S transition and new origin firing. On the other hand, HOXC10 activates NF-kb, G2/M checkpoint and DNA repair through NER pathway, protecting cells from apoptosis and DNA damage, especially DNA crosslinks. Interestingly, HOXC10 functions at later stage of DNA damage response, mainly at maintenance of the checkpoint until repair is complete. This eventually leads the cells to become less sensitive to chemotherapy treatment. Most importantly, cells selected to become resistant to drug treatment over long time drug exposure show a significant increase in HOXC10 expression. In conclusion, in this report we show for the first time that HOXC10 upregulation in breast cancer may have some clinical implications since it affects tumor growth, genomic instability and response to some chemotherapy drugs.					
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Introduction:

HOX genes have been well described as important players in development and morphogenesis, and more recently, in carcinogenesis. Work from our lab and others has shown that some of these genes have important role in breast cancer progression and metastasis, since they control processes such as angiogenesis, DNA repair, apoptosis, and migration. To better identify the key HOX players in breast cancer, we conducted a HOX tiling array, and found that HOXC10 was among the most significantly overexpressed genes in primary and metastatic breast tumors compared to normal tissue. Therefore, in this proposal, we are addressing the function of HOXC10 upregulation on breast cancer progression and drug resistance.

Body:

Task1. To determine the transforming effect of HOXC10 overexpression in cancer breast cell lines using in vivo and in vitro approaches

Summary of previously reported data:

In the last report, we have shown that stably overexpressing or knocking-down HOXC10 levels in some breast cancer cell lines affected invasion, anchorage-independent growth and proliferation, especially under low growth factor conditions in vitro. When assessed in vivo, xenografts with high HOXC10 expression grew faster, were more vascularized and expressed elevated levels of chemokines and cytokines.

Progress:

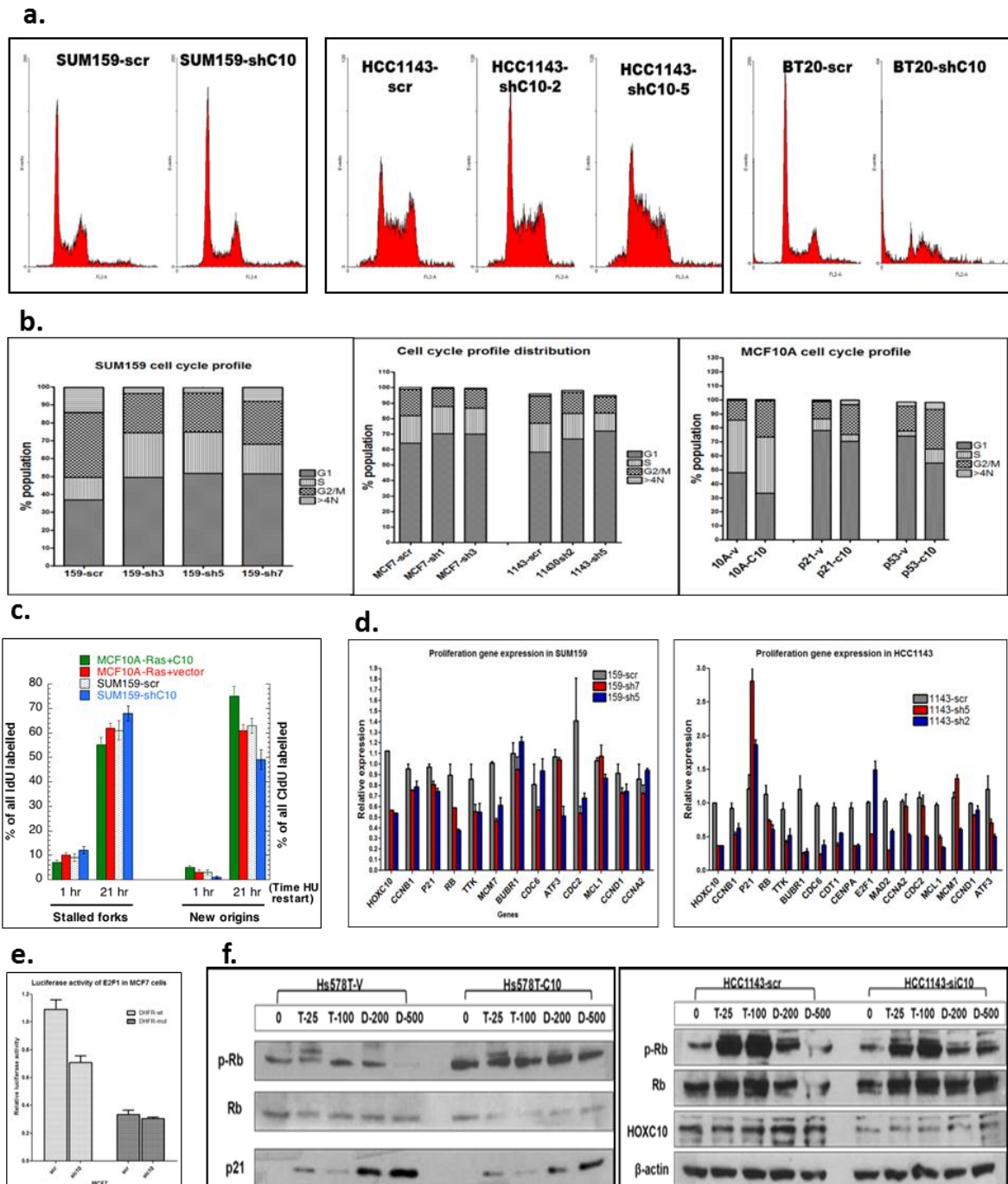
Using in-vitro systems of stable or transient knockdown or overexpression, the role of HOXC10 in breast cancer proliferation was further investigated.

- A. Different breast cancer cell lines with stable HOXC10 knockdown (by shRNA) were established. Cell cycle analysis (PI staining) shows that upon loss of HOXC10, cells tend to arrest at G1 or S phase (Fig 1a, b). On the other hand, stably overexpressing HOXC10 in MCF10A, and its 2 knockout variants, p21^{-/-} and p53^{-/-}, led to a decrease in the G1 population (Fig 1b). Note that these results also suggest that HOXC10 role in proliferation is independent of p53 pathway.
- B. To obtain a better understanding of HOXC10 role during DNA replication, DNA fiber analysis was utilized to monitor replication perturbation (genome-wide) at single-molecule resolution (collaboration with Dr Tej Pandita). Results show that HOXC10 doesn't affect the stability of stalled forks but its expression led to more new origin firing, explaining partially the increase in proliferation (Fig 1c).
- C. Since Rb/E2F1 pathway is one of the major pathways controlling G1/S transition, proliferation and replication, we next investigated if HOXC10 modulates it. Indeed, knocking-down its expression in SUM159 or HCC1143 reduces the expression of many E2F1 target genes at the RNA or protein levels, while the overexpression of HOXC10 slightly increases their expression (Fig 1d). A luciferase construct with E2F responsive element (in DHFR promoter) further confirmed that reduction of HOXC10 expression decreases E2F1 activity (Fig 1e).
- D. Finally, a correlation between HOXC10 expression and Rb phosphorylation/deactivation was observed by western (especially after drug treatment, Fig 1f), suggesting that HOXC10 might

modulate the activity of G1 CDKs or CDKis which eventually control the Rb/E2F pathway. Further investigation is needed to identify the direct target of HOXC10.

Figure 1.

A. SUM159, HCC1143 and BT20 stably knocking-down HOXC10 by shRNA were established. Cell cycle was analyzed by propidium iodide. Representative graphs are shown. B. Along with the above cell lines, MCF10A and its 2 knockout variants, p21^{-/-} and p53^{-/-}, were established to overexpress HOXC10 exogenously. After cell cycle analysis through propidium iodide, the percentage of cells in different phases was quantified. Shown is the average of at least 2 independent repeats. C. DNA fiber analysis of MCF10A-Ras-v/C10 and SUM159-scr/shC10 showing that there is a defect in new origin formation in low HOXC10 cells. D. RNA was collected from SUM159-scr, HCC1143-scr and 2 of their shC10 clones. The expression of many of E2F1 target genes was analyzed by qRT-PCR. The average of 3 repeats is shown. E. MCF7-scr or shC10 were transfected with a wild-type or mutant DHFR-promoter construct. Luciferase activity was quantified and the average was calculated from 3 experiments. E. HS578T-v/C10 and HCC1143-scr/shC10 were treated with doxorubicin or taxol for 24h. Protein was collected and analyzed for the expression of pRB.



Task2. To determine the downstream pathway(s) through which HOXC10 functions to decrease response to chemotherapy treatment

Summary of previously reported data:

We have previously shown that HOXC10 might activate EGFR and PI3k/AKT pathways, which are important for cancer cell survival. This led us to investigate if HOXC10 affects response to chemotherapy treatment and we therefore found that cell lines with high HOXC10 expression have reduced sensitivity to many drug classes, and show a decrease in apoptosis upon treatment.

Progress:

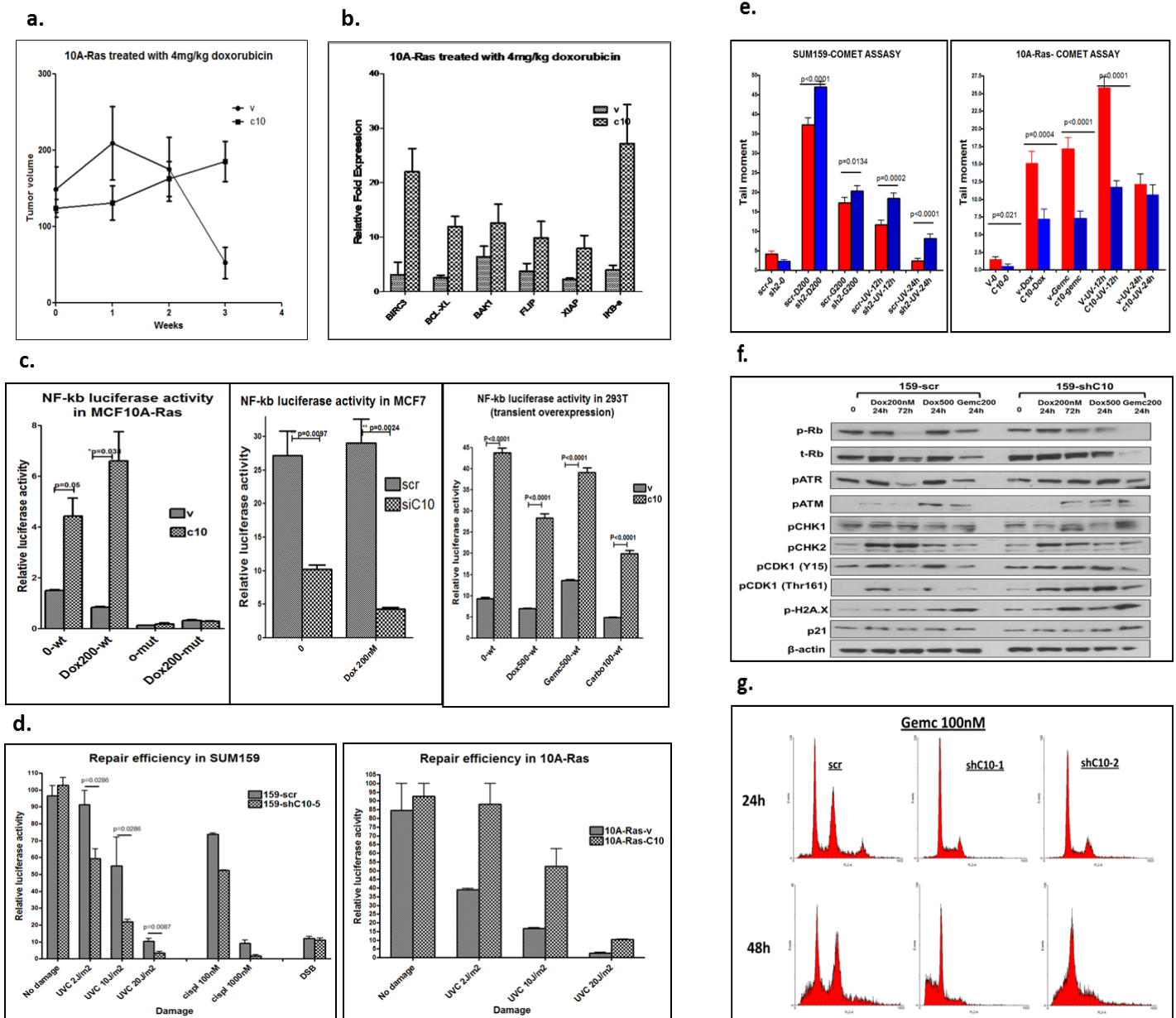
- A. First, we confirmed the previous in vitro data by establishing xenografts of MCF10A-Ras that overexpress an empty vector or HOXC10. When treated with doxorubicin, 10A-Ras-C10 tumors stopped growing at the beginning but eventually relapsed. Further, they display an increase in many anti-apoptotic genes compared to 10A-Ras-V tumors (Fig 2a, b).
- B. Since NF- κ B is a common downstream effector of many survival pathways and that many genes modulated by HOXC10 expression are known targets of NF- κ B, we checked its activity by a luciferase system. Results confirm that HOXC10 activates NF- κ B, especially after drug treatment (Fig 2c).
However, the effect on NF- κ B was only part of the mechanism for survival in HOXC10 overexpressing cells, since inhibiting this pathway by drug didn't reverse completely the phenotype (data not shown).
- C. We next investigated if DNA damage repair is modulated by HOXC10. Using a host cell reactivation assay, we found that HOXC10 expression improves DNA damage repair against DNA crosslinks (Fig 2d). Furthermore, no such effect was seen in double-strand breaks or homologous recombination assays (data not shown), suggesting that HOXC10 activates mainly the nucleotide excision repair (NER) pathway.
- D. We next conducted the alkaline comet assay. Consistent with the above data, there was a significant decrease in the tail moment after drug or UV treatment in cells with high HOXC10 expression (Fig 2e).
- E. Interestingly, the initial induction of DNA damage response pathways were not affected by the expression of HOXC10, but the long-term maintenance of their activity was higher (Fig 2f and data not shown). Also, those cells were more efficiently arrested at the G2 checkpoint over days, and didn't proceed in the cell cycle and undergo apoptosis as compared to their counterpart with low HOXC10 levels (Fig 2g).

All these data suggests that HOXC10 is involved in later steps in the DNA repair pathway to halt the progression of the cells until repair is complete, and therefore prevent apoptosis and mitotic catastrophe.

As part of task 3 and 4 of the SOM, studies are currently being conducted to discover the protein that HOXC10 interacts with to induce proliferation and DNA damage repair. Also, a set of microRNA mimics are being tested as a novel way to inhibit HOXC10 overexpressing tumors.

Figure 2.

A. MCF10A-Ras-v or overexpressing HOXC10 were established in nude mice, and then treated with 4mg/kg doxorubicin (3x, once per week; 4 tumors per group). Tumor growth was monitored during treatment. B. Mice were sacrificed one week after the last injection. RNA was extracted and the expression of some apoptotic genes was measured by qRT-PCR. C. MCF10A-Ras stably expressing HOXC10, MCF7 stably expressing shRNA-HOXC10 or 293T with transient transfection with HOXC10, were transfected with a luciferase construct containing NF- κ B responsive elements. Cells were treated with the appropriate drug for 24h before luciferase activity was quantified. The experiment was repeated at least 3 times, and t-test was used for the statistical analysis. D. Host reactivation assay in MCF10A-Ras-v/C10 and SUM159-scr/shC10. pGL3-promoter was treated with cisplatin for 16h or was irradiated with UVC to induce crosslinks. Damaged plasmid was transfected into cells and luciferase activity was measured as a reflection of the repair efficiency of the different cells. Data represent at least 3 independent measurements, and t-test was used for statistical analysis. E. Cells were treated with the appropriate drug or UVC for 12h or 24h. Alkaline comet assay was performed to check the extent of DNA damage. At least 100 nuclei were quantified, and results were analysis using t-test. F. SUM159-scr/shC10 were treated with doxorubicin or gemcitabine as indicated. Protein was collected and the phosphorylation level of many proteins involved in DNA damage repair was examined. G. SUM159-scr and 2 shC10 cells were treated with gemcitabine 100nM. Cells were collected for PI staining analysis at 24h and 72h.



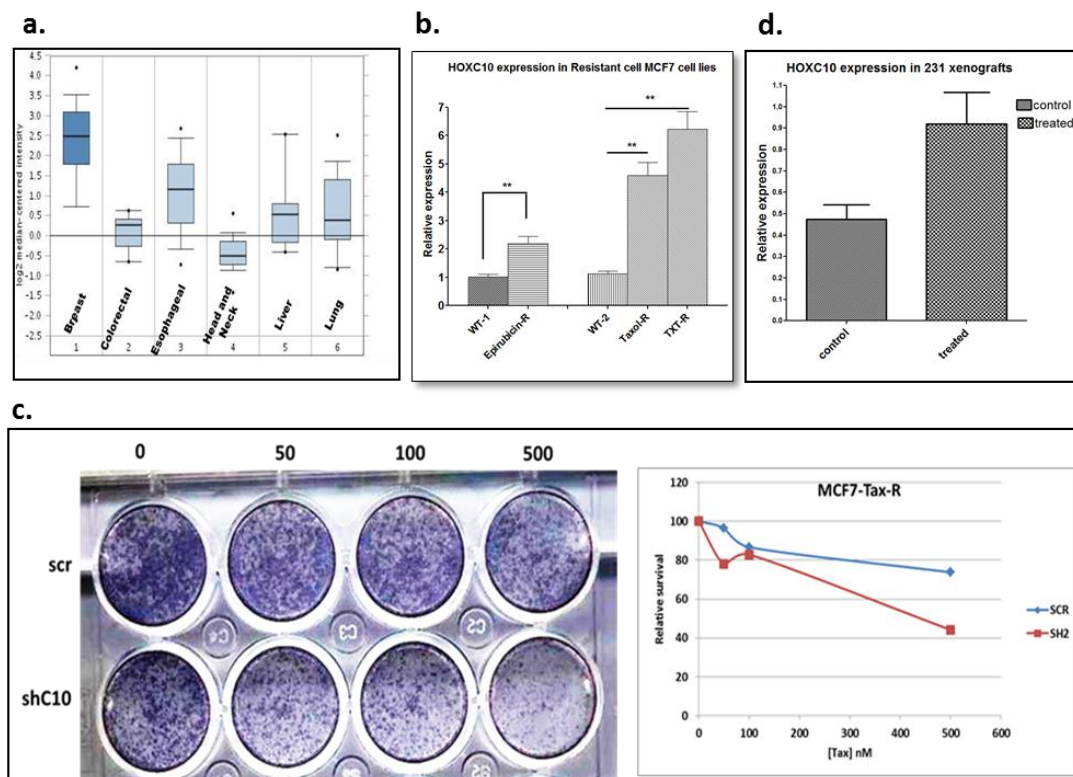
Task3. To determine the value of HOXC10 expression as a prognostic and diagnostic marker

Progress:

- Mining the online databases like “Oncomine” and “NexBio” revealed a significant upregulation of HOXC10 in breast tumors. Interestingly, this was selective for breast cancer, as other cancer types did not display the same pattern (Fig 3a).
- As anticipated, HOXC10 overexpression was seen in all the different grades and subtypes (maybe because metastasis may be an inherent property of the tumor, even from early stages), and therefore could not be used as a potential biomarker for aggressiveness (data not shown). Efforts are being instead conducted to check if HOXC10 expression has a prognostic value for response to some chemotherapy treatment regimens in patients.
- Using a panel of in vitro drug resistance cell lines (MCF7 parental and its sublines- epirubicin, taxol and docetaxel resistant- developed by Dr Parissenti), we found that HOXC10 expression increases upon acquiring resistance (Fig 3b).
- When HOXC10 levels were reduced in some of these resistant sublines, resensitization of the cell lines to drug treatment was observed (Fig 3c).
- Finally, MDA-MB-231 xenografts were established in mice and treated either with doxorubicin, carboplatin or taxol. Tumors resistant to drug had a higher expression of HOXC10 (Fig 3d).

Figure 3.

A. HOXC10 is preferentially overexpressed in breast cancers. Patient data was retrieved and visualized through Oncomine™ (Compendia Bioscience, Ann Arbor, MI). p-value= 9.1 e-40, t-test: 16.731, Fold change: 4.418. B. MCF7 parental and resistant sublines were received from Dr Parissenti. RNA was extracted and the expression of HOXC10 was measured by qRT-PCR. ** p<0.001 (t-test). C. MCF7-taxol-R with HOXC10 knockdown were established, and tested for resensitization to taxol treatment by colony survival assay (crystal violet staining (left) and quantification (right)). D. MDA-MB-231 xenografts were established in mice and treated either with doxorubicin, carboplatin or taxol (once per week, for 4 weeks). Resistant tumors to drug were collected one week after the last treatment, RNA was extracted and HOXC10 expression was quantified by qRT-PCR. p=0.056 (t-test).



Key Research Accomplishments:

- HOXC10 overexpressing cells (endogenously or exogenously) were proliferating faster, were less arrested at G0/G1, and had more origin firing as compared to their low HOXC10-expressing counterparts. This was due at least to a role of HOXC10 in activating the Rb/E2F1 pathway, by modulating Rb phosphorylation.
- By decreasing apoptosis, activating NF-kB pathway and enhancing the DNA repair pathway (mainly NER pathway), HOXC10 decreases susceptibility of the cells to chemotherapy treatment.
- HOXC10 is involved in later steps in DNA damage response, arresting the cells at the G2 checkpoint until repair is complete.
- Breast cancer cell lines progressively selected to become resistant to some chemo drugs display a higher HOXC10 expression.

Reportable Outcomes:

Poster will be presented at:

Sadik H, Nguyen Nguyen, Rakesh Kumar, Tej Pandita, Sukumar S (December 2012). HOXC10, a Homeobox protein Overexpressed in Breast Cancer, modulates the response to Chemotherapy treatment. San Antonio Breast Cancer Symposium (SABCS), San Antonio, TX

Conclusion:

As a clear involvement of the HOX family in carcinogenesis has been accumulating over the years, we took a high-throughput approach on the HOX loci and found that HOXC10 is one of the most significantly overexpressed genes in breast tumors as compared to normal tissues. This proposal addresses for the first time the function of HOXC10 in breast cancer tumorigenicity and drug resistance.

As an expansion to my previous work, I found that HOXC10 downregulation led to a decrease in E2F1 activity due to a reduction in Rb dephosphorylation especially after treatment with chemodrugs. This was reflected by a decrease in proliferation rate, to an arrest in G0/G1 phase and to a reduction in new origin firing. However it is still unknown whether HOXC10 interacts directly with a G1/S CDK (CDK4/6/2) or with a CDK inhibitor (p16, p21...) to affect Rb dephosphorylation. Further studies are currently undergoing to address this question.

As I had previously shown that HOXC10 overexpression allows the cells to become less sensitive to different chemotherapy drugs, I investigated the pathway through which HOXC10 functions in DNA damage response, and found that first, it activates NF-kB allowing the cells to be less prone to apoptosis. Second, it activates DNA repair, mainly the NER pathway. And finally, it maintains the G2/M checkpoint to arrest the cells and allow them to complete their repair before proceeding in the cell cycle and undergoing mitotic catastrophe. Future work revolves on finding the interacting partner of HOXC10 that drives chemo-desensitization. Also I am testing a panel of microRNAs that can reverse the phenotype of HOXC10 overexpression as a novel strategy to attack those tumors.

Lastly, the more direct outcome of my work is to investigate if HOXC10 can be used as a prognostic or diagnostic marker. Preliminary data with in vitro and in vivo system show that HOXC10 expression is higher in tumors/cell lines resistant to drug treatment. However, mining the online databases for HOXC10 expression in treated patients with known outcome will be the most important evidence for the value of HOXC10 as a prognostic marker.

Training:

During the reported period, Dr Sukumar along with my thesis committee were monitoring my progress and guiding me through completion of the tasks of my thesis and my grant. I am also having the opportunity to attend a conference for breast cancer and present a poster. Weekly meetings and conferences (every Tuesday, Wednesday and Friday) from scientists at Hopkins or outside were of great value. Further, I am having great opportunities to collaborate with experts in DNA damage experts in and outside Johns Hopkins University, and with microRNA biotech company. Also, all the research facilities are provided within a walking distance and are available all the time. Finally, the lab environment is so rich with post-docs and pre-docs with different skills and background, which helped me master basic methods and more complicated techniques such as flow cytometry and in vivo work. In sum, inside and outside supports are continuously available for me to complete the task of my grant.

Appendices:

Sadik H, Nguyen Nguyen, Rakesh Kumar, Tej Pandita, Sukumar S (December 2012). HOXC10, a Homeobox protein Overexpressed in Breast Cancer, modulates the response to Chemotherapy treatment. San Antonio Breast Cancer Symposium (SABCS), San Antonio, TX

Background: Breast cancer is the second leading cause of cancer deaths in women worldwide. Although chemotherapy is effective, resistance to drugs develops over time and can account for treatment failure in over 90% of metastatic breast cancer patients. HOX genes are homeobox-containing transcription factors well-known for their role in morphogenesis. However, accumulating evidence has emphasized their importance during carcinogenesis and metastasis. The goal of this study is to understand the role of HOXC10 in breast cancer and the consequence of its overexpression in the response to chemotherapy.

Methods: Using a tiling array of all four HOX clusters in a panel of primary and metastatic breast cancer tissues, we identified HOXC10 as being among the highly overexpressed genes in breast cancer. Then using a panel of cell lines that either stably overexpress exogenous HOXC10 or cell lines with stably downregulated endogenous HOXC10 (mediated by shRNA), we investigated the role of HOXC10 in proliferation, response to chemotherapy treatment and repair of DNA damage.

Results: HOXC10 is overexpressed in 67% of primary breast tumors (n=31), in 82% of the metastatic tissues (n=49) and in most breast cancer cell lines (n=48). In vitro and in vivo investigation confirmed that HOXC10 plays an oncogenic role in breast cancer. Further, knockdown of HOXC10 in a panel of breast cancer cell lines slowed their proliferation and arrested them at the G1 phase, by inactivating the RB/E2F pathway, decreasing the number of new origins and eventually reducing the polyploidy population.

Cell survival assays after different chemotherapeutic drug treatment showed that overexpression of the exogenous HOXC10 in MCF10A led to less susceptibility to most drugs. This was partially due to a protection from apoptosis by upregulating and activating the anti-apoptotic machinery such as the NF-kb pathway. Further investigation revealed the involvement of HOXC10 in DNA repair (and not initial response), especially after DNA crosslink damage. Interestingly, the binding of HOXC10 to CDK7 in a region outside its homeodomain activates CDK7 activity towards RNA polymerase II mainly in response to DNA damage. Since HOX genes are difficult to target therapeutically, one potential approach to overcome chemoresistance in HOXC10 overexpressing cells is by including CDK7 inhibitors (already in clinical trials).

All these results were confirmed in the SUM159 model which stably expresses a HOXC10-shRNA.

Finally, HOXC10 was found to be significantly overexpressed in MCF7 isogenic cell lines gradually selected to be resistant to some chemotherapeutic drugs. By knocking down HOXC10 in these sublines, resistance to the drug was reduced. Further, SUM159 and MDAMD231 xenografts that were treated with chemotherapy over weeks and that show partial to no response tend to have a higher expression of HOXC10.

Conclusion: This study shows that HOXC10, a homeobox protein previously shown to be regulated during the cell cycle and to have a positive effect on proliferation, is overexpressed in the majority of breast cancers. This upregulation may have clinical implications since cells with higher expression of HOXC10 tend to have more genomic instability and activation of anti-apoptotic and DNA repair pathways, which eventually modulate the response to some chemotherapy drugs.